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Regulation of the Expression of the Glycine Decarboxylase Complex during Pea Leaf Development

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The expression of the genes encoding the four proteins (P, H, T, and L) of glycine decarboxylase, a multienzymatic complex involved in the mitochondrial step of the photorespiration pathway, was examined during pea (*Pisum sativum*) leaf development in comparison with ribulose-1,5-bisphosphate carboxylase/oxygenase. Mitochondria from the primary leaf were isolated at several well-defined stages of development. Their capacity to oxidize glycine was negligible during the earlier stages but increased dramatically once the leaflet opened. This was correlated with the accumulation of the glycine decarboxylase complex (GDC) proteins, which was shown to occur in preexisting mitochondria, producing an increase in their density. The transcription of the GDC genes was coordinated and occurred early, with a peak at 7 d, a stage at which mitochondria are unable to oxidize glycine. This implies the existence of posttranscriptional control of gene expression. The comparison of the expression patterns of the genes encoding specific proteins of GDC with that of *rbcS* genes suggests a common regulation scheme that is related to light induction. However, ribulose-1,5-bisphosphate carboxylase/oxygenase is present in the chloroplast well before GDC fills the mitochondria, suggesting that the setup of photorespiration occurs in cells already engaged in active photosynthesis.

GDC (or the Gly cleavage system) catalyzes the oxidative decarboxylation and deamination of Gly into CO_2, NH_3, NADH, and N^5N^{10}-methylene-5,6,7,8-tetrahydropteroyl-polyglutamic acid. This enzymatic system is present in bacteria, including *Peptococcus glycinophilus* (Klein and Sagers, 1966), *Arthrobacter globiformis* (Kochi and Kikuchi, 1969), and *Escherichia coli* (Okamura-Ikeda et al., 1993), as well as in eukaryotic cells (Kikuchi and Hiraga, 1982; Oliver et al., 1990; Douce et al., 1994). In mammals GDC, which represents a minute fraction of the total soluble mitochondrial protein, is involved in the major pathway of catabolic degradation of Gly (Yoshida and Kikuchi, 1971). In plants GDC is localized in leaf mitochondria, where it catalyzes, in association with SHMT, the mitochondrial step of the photorespiratory pathway (Neuburger and Douce, 1977; Lorimer and Andrews, 1981; Husic et al., 1987).

GDC has been purified from plants (Neuburger et al., 1986; Walker and Oliver, 1986a; Bourguignon et al., 1988) and, like its mammalian or bacterial counterpart, it is composed of four different component proteins that are referred to as the P-, H-, T-, and L-proteins. The P-protein (a homodimer of 105-kD peptides), which binds the α-amino group of Gly through its pyridoxal phosphate cofactor, catalyzes the decarboxylation of Gly in the presence of the H-protein (a monomer of 14 kD). The carboxyl carbon is released as CO_2, and the remaining methylamine moiety of Gly is transferred onto the lipoamide arm of the H-protein. The lipoamide-bound methylamine group is shuttled to the T-protein (a monomer of 41 kD) while the methylene carbon is transferred to 5,6,7,8-tetrahydropteroyl-polyglutamic acid to produce N^5N^{10}-methylene-5,6,7,8-tetrahydropteroyl-polyglutamic acid and the amino nitrogen is released as NH_3. Finally, the L-protein (a dimer of 50 kD) reoxidizes the dihydrolipoamide of the H-protein by the sequential reduction of FAD and NAD^+. In plants P-, H-, T-, and L-proteins are synthesized from nuclear-encoded genes (Walker and Oliver, 1986b), which have been mapped on the pea (*Pisum sativum*) chromosomes (Turner et al., 1993). All of the cDNAs corresponding to these proteins have been isolated and characterized (Kim and Oliver, 1990; Macherel et al., 1990; Bourguignon et al., 1992, 1993; Turner et al., 1992a, 1992b). Mitochondria isolated from etiolated pea leaves oxidized Gly at very low rates, whereas mitochondria from green leaves exhibited high rates of Gly oxidation and this was correlated to the presence in large amounts of the proteins of GDC (Day et al., 1985; Walker and Oliver, 1986b). Northern and western blot analyses of the expression of the GDC proteins in pea revealed that the P-, H-, and T-proteins were expressed predominantly in the leaf tissue (Kim and Oliver, 1990; Macherel et al., 1990; Kim et al., 1991; Turner et al., 1992a; Bourguignon et al., 1993). However, in contrast to these three proteins, the expression of the L-protein occurs in all of the tissue examined so far. In fact, this dihydrolipoamide dehydrogenase is present in all of the tissues because it is also the component of other mitochondrial complexes, in

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Abbreviations: GDC, Gly decarboxylase complex; SHMT, Ser hydroxymethyltransferase.

particular the pyruvate dehydrogenase complex (Bourguignon et al., 1992, 1996; Turner et al., 1992b).

The accumulation of GDC proteins in mitochondria from green leaves could be attributed to an increase in mRNA levels for P-, H-, and T-proteins (Kim and Oliver, 1990; Macherel et al., 1990; Turner et al., 1992b), and a light-dependent transcriptional control of the genes encoding these proteins was suggested (Srinivasan et al., 1992). Recently, several light-responsive elements (GT boxes) have been characterized in the promoter region of the *gdcH* gene from *Arabidopsis thaliana* (Srinivasan and Oliver, 1995), and it is tempting to parallel the accumulation of GDC in the matrix of mitochondria with the accumulation of Rubisco in the stroma of the chloroplast because both enzyme complexes reach millimolar concentrations when plants are grown in light conditions. We recently detected several *cis*-acting elements in the promoter region of the *gdcT* gene of pea, which could interact with the same nuclear factors involved in the regulation of the *rbcS* and *cab* genes (Gilmartin et al., 1990).

In the present study we investigated the biogenesis of GDC in comparison with that of Rubisco during the development of pea. For this purpose we isolated the mitochondria from leaves taken at several well-defined stages of development to measure their capacity to oxidize Gly and the content of individual proteins of GDC. Chloroplasts were also isolated to follow Rubisco activity during development. Finally, mRNA levels for GDC proteins and Rubisco were determined by northern blotting. The results of this detailed analysis are discussed to sketch out the biogenesis of GDC during pea development.

MATERIALS AND METHODS

Seeds of pea (*Pisum sativum* var Douce Provence) were allowed to imbibe in running tap water overnight and were planted in trays of vermiculite in a growth cabinet with a light period of 14 h and a dark period of 10 h. As shown in Figure 1, the first two leaflets of each plant were harvested at different developmental stages (4 d, L1; 5 d, L2; 7 d, L3; 9 d, L4; 12 d, L5 and L'5) to prepare mitochondria, chloroplasts, and RNA.

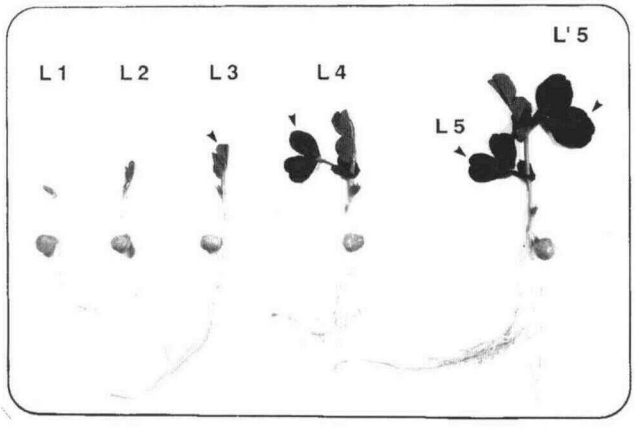

Figure 1. The different stages of pea leaf development used to study the biogenesis of the Gly decarboxylase. L1, L2, L3, L4, and L5 correspond to the developmental stages of the primary leaf of 4-, 5-, 7-, 9-, and 12-d-old plants. L'5 corresponds to the developmental stage of the second leaf of 12-d-old plants. Arrowheads indicate leaves harvested.

Isolation of Mitochondria

Pea leaflets (50–100 g) from each developmental stage (L1–L5 and L'5 as described in Fig. 1) or from 12-d-old etiolated plants were cut in 200 to 300 mL of chilled extracting medium containing 20 mM pyrophosphate, pH 7.5, 0.3 M mannitol, 3 mM 2-mercaptoethanol, 1 mM EDTA, 0.1% (w/v) BSA (fraction V, Boehringer Mannheim), and 0.5% (w/v) PVP25. All of the procedures described below were carried out at 4°C. The plant tissue was disrupted at a low speed for 3 to 5 s in a 1-L Waring blender. The brei was rapidly squeezed through eight layers of muslin and 50-μm nylon mesh. The filtered suspension was then centrifuged at 3,000 rpm for 15 min (GS3 rotor, Sorvall, Dupont). The pellet that was kept on ice was used to isolate the chloroplasts as described in the next paragraph. The supernatant was centrifuged for 20 min at 8,000 rpm (GS3 rotor, Sorvall). The pellet was then resuspended, using a paintbrush, in 10 mL of wash medium containing 10 mM K_2HPO_4 (pH 7.4), 0.3 M mannitol, 1 mM EDTA, and 0.1% (w/v) BSA. The aggregates were disintegrated using a glass Potter-Elvehjem homogenizer (CONNIE, Gattinkon, Switzerland) with two to three gentle "up-and-down" strokes. This mitochondrial suspension was then centrifuged at 2,500 rpm for 10 min (SS34 rotor, Sorvall) to pellet the remaining heavy cell organelles. The pellet was discarded and the supernatant was centrifuged for 20 min at 12,000 rpm (SS34 rotor, Sorvall). The pellet containing washed mitochondria was gently resuspended in a small volume (1–2 mL) of wash medium. Two different protocols were then used to purify the mitochondria.

The washed mitochondria isolated from leaflets of 4-, 5-, and 7-d-old green pea plants were purified using self-generated Percoll gradients, essentially as described by Neuburger et al. (1982), for the purification of potato tuber mitochondria. The resuspended washed mitochondria (2–3 mL) were layered on 33 mL of Percoll medium containing 10 mM K_2HPO_4 (pH 7.2), 0.3 M Suc, 1 mM EDTA, 0.1% BSA, and 28% (v/v) Percoll. The tubes were then centrifuged for 50 min at 18,000 rpm (SS34 rotor, Sorvall) by using the automatic rate controller. After the centrifugation, the mitochondria were found in a tight, brown band in the middle of the tube and the thylakoids remained near the top of the tube. The purified mitochondria, collected with a Pasteur pipette, were diluted 8-fold with the wash medium without BSA and centrifuged at 12,000 rpm for 20 min (SS34 rotor, Sorvall). The mitochondria were washed again with the same medium, pelleted at 12,000 rpm for 20 min (SS34 rotor, Sorvall), and resuspended in a small volume of wash medium without BSA.

The washed mitochondria isolated from leaflets of 9- and 12-d-old green pea plants were purified using a method that employs a self-generating gradient of Percoll in combination with a linear gradient of PVP25 (0–10% [w/v]), of Suc (0.3–0 M), and of raffinose (0–0.3 M) in the manner

described by Douce et al. (1987). The tubes were then centrifuged for 50 min at 18,000 rpm (SS34 rotor, Sorvall) using the automatic rate controller. After centrifugation, the mitochondria were found in a tight, brown band near the bottom of the tube, and the purified mitochondria were then washed as described above.

To compare the density of the mitochondria that were isolated from different developmental stages, we performed a linear gradient of Percoll (28 to 42% from top to bottom of the tube) in the presence of 0.3 M Suc, 10 mM K_2HPO_4, pH 7.4, 1 mM EDTA, and 0.1% (w/v) BSA in a total volume of 33 mL. Three milliliters of washed mitochondria (approximately 18 mg) that were isolated from etiolated pea leaves, from leaflets of 7-d-old green plants, or from leaflets of 12-d-old green plants were loaded on the Percoll gradient. Pharmacia marker beads were also loaded on a separate Percoll gradient to measure the density along the gradient. The tubes were then centrifuged for 50 min at 18,000 rpm (SS34 rotor, Sorvall) using the automatic rate controller. The gradients were then fractionated in 2-mL fractions. The 18 fractions thus obtained were diluted 15-fold with the wash medium and centrifuged at 12,000 rpm for 20 min (SS34 rotor, Sorvall). The supernatant was aspirated and each pellet was resuspended in a small volume of wash medium. The final volume of the suspension was between 100 and 400 μL. One-fifth to one-tenth of the volume was used to measure the mitochondrial NADH oxidation with the Clark-type O_2 electrode system purchased from Hansatech (Norfolk, UK).

Measurements of Substrate Oxidation by Mitochondria

Mitochondrial O_2 uptake was measured in the presence of different substrates (NADH, pyruvate, malate, and Gly) at 25°C using the Clark-type O_2 electrode system. The reaction medium contained 20 mM K_2HPO_4, pH 7.3, 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1% BSA, and known amounts of mitochondrial protein in a total volume of 1 mL. The O_2 concentration in air-saturated medium was taken as 240 μM.

NADH oxidation was measured in the presence of 2 mM NADH and aliquots of ADP (100 nmol) (state III) added to the the reaction medium. Pyruvate oxidation was measured in the reaction medium in the presence of 10 mM pyruvate, 100 μM thiamine pyrophosphate, 0.2 mM NAD^+, 0.4 mM CoA, 0.5 mM malate, and aliquots of ADP (100 nmol). Malate oxidation was assayed in the presence of 10 mM malate, 0.2 mM NAD^+, and aliquots of ADP (100 nmol) added to the reaction medium. Gly oxidation was measured in the presence of 10 mM Gly, 0.2 mM NAD^+, and aliquots of ADP (100 nmol) added to the reaction medium.

Isolation of Chloroplasts and Measurement of Rubisco Activity

The first pellet obtained during the isolation of the mitochondria, which contained the chloroplasts, was resuspended in 5 mL of medium of the following composition: 0.33 M mannitol, 30 mM Hepes/KOH, pH 7.5, 2 mM EDTA, and 0.1% BSA. This suspension was filtered on a nylon mesh (pore size of 50 μm) and intact chloroplasts were purified using a Percoll gradient according to the method of Mourioux and Douce (1981). Rubisco activity was measured according to Lorimer (1982).

Protein Determination, SDS-PAGE, and Immunoblot Analyses

Protein determinations were performed according to Lowry et al. (1951). The organelle suspension was first solubilized in 0.4% deoxycholate, and for each sample three protein determinations were carried out using BSA as a standard. Proteins were separated by SDS-PAGE using conventional techniques. The proteins were electrophoretically transferred onto nitrocellulose sheets (Bio-Rad) using the semidry Transblot SD apparatus (Bio-Rad). The immunoblotting detection was performed with polyclonal antibodies against the mitochondrial H-, T-, P-, and L-proteins and SHMT as described by Bourguignon et al. (1992).

RNA Extraction and Northern Blot Analyses

RNA extraction was performed as described by Macherel et al. (1990) using 3 to 5 g of leaflets from the different developmental stages, as described earlier in the text. The cDNAs encoding the H-, L-, and T-proteins that were isolated by Macherel et al. (1990) and Bourguignon et al. (1992, 1993) were used as probes for northern blot analysis. cDNA probes encoding the P-protein and rbcS 3A were amplified by PCR from a pea cDNA library in λ gt11 (Macherel et al., 1990) using specific oligonucleotides designed from the sequence of the corresponding cDNAs published by Turner et al. (1992a) and Coruzzi et al. (1983). Labeling of probes, RNA gel separation, blotting to nylon membrane, and hybridization were performed as described previously (Macherel et al., 1990). The quantification of the signals was performed with a phosphor imager apparatus (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Gly Oxidation by Mitochondria Isolated at Various Stages of Pea Leaf Development

The capacity of pea leaf mitochondria to oxidize Gly was examined during pea development. Mitochondria were isolated from pea leaves that were harvested at the different developmental stages (4 d, L1; 5 d, L2; 7 d, L3; 9 d, L4; and 12 d, L5 and L'5) presented in Figure 1. The primary (thus oldest) leaf was selected at all stages, except at stage L'5, where the second leaf was also harvested. Two different types of gradients were used, depending on the stages of development. For the 4-, 5-, and 7-d-old plants, we used a self-generating 28% Percoll gradient that is generally used for the purification of mitochondria from potato tubers. On the other hand, for 9- and 12-d-old plants we used a self-generating 28% Percoll gradient in combination with a linear gradient of PVP25, Suc, and raffinose. This type of gradient is generally used to purify mitochondria from green tissues (Douce et al., 1987).

Mitochondria that were isolated from the earlier developmental stages (L1, L2, and L3, corresponding to 4-, 5-,

and 7-d-old plants) oxidize Gly at very low rates (3.2–12 nmol O_2 consumed min^{-1} mg^{-1} mitochondrial protein), and addition of ADP did not trigger an increase in the rate of O_2 consumption (Table I). However, a rapid Gly oxidation is observed in the presence of ADP (138–232 nmol O_2 consumed min^{-1} mg^{-1} mitochondrial protein) when mitochondria are isolated from the latter stages (L4, L5, or L'5). In contrast to Gly, other substrates, such as pyruvate, malate, or NADH, are oxidized (in the presence of ADP) very efficiently at all of the stages of pea leaf development (Table I).

Accumulation of Gly Decarboxylase in Mitochondria during Pea Leaf Development

Total mitochondrial proteins were subjected to SDS-PAGE analysis and immunological detection using antibodies raised against the P-, T-, H-, and L-proteins of GDC and against SHMT. Figure 2 shows that the specific polypeptides of GDC (P, T, and H) and SHMT are present in a small amount in the mitochondria that were isolated at the early stages of pea leaf development (L1, L2, and L3), whereas at stages L4, L5, and L'5 a massive accumulation of all of these proteins was observed in the mitochondria. In the case of L-protein, we found that a substantial amount of protein was present in the earlier stages and that there was only a slight increase in the later stages.

Increase of the Density of the Mitochondria during Pea Leaf Development

In contrast to mitochondria isolated from mature leaves, mitochondria isolated from young pea leaves (4-, 5-, and 7-d-old plants) were unable to penetrate into the self-generated Percoll gradient that was generally used. However, the same mitochondria readily penetrated into the Percoll gradient that was used for the purification of the mitochondria from potato tubers. We suspected, therefore, that the mitochondria isolated from young pea leaves were lighter in density than the mitochondria isolated from older leaves. To evaluate such a change in density, we decided to compare the density of the mitochondria isolated from pea leaves at the stages L3 (7 d) and L5 (12 d) with the mitochondria isolated from etiolated leaves (12 d). We isolated mitochondria from the three types of leaves by differential centrifugation and loaded the suspensions on the top of 28 to 42% linear Percoll gradients. After isopycnic centrifugation, the gradients were fractionated. The detection of mitochondria in the fractions was done by measuring the state 3 rate of NADH oxidation using a classical oxygen electrode. In the case of young green leaves (7-d-old plants) or etiolated leaves (12-d-old plants), the bulk of the mitochondria remained in the top part of the gradient (Fig. 3), and, in marked contrast, the mitochondria isolated from mature leaves (12-d-old plants) were found in the bottom part of the gradient (Fig. 3). The apparent density of the mitochondria isolated from mature leaves, estimated with the density marker beads (Pharmacia) in our experimental conditions, is around 1.096, which is higher than the density of 1.049 measured for the mitochondria isolated from etiolated or young leaves. It is interesting that cell organelles isolated from whole pea leaflets containing leaves of various ages yield a mixed population of mitochondria, leading to the impression that two distinct populations of mitochondria exist in leaf tissues.

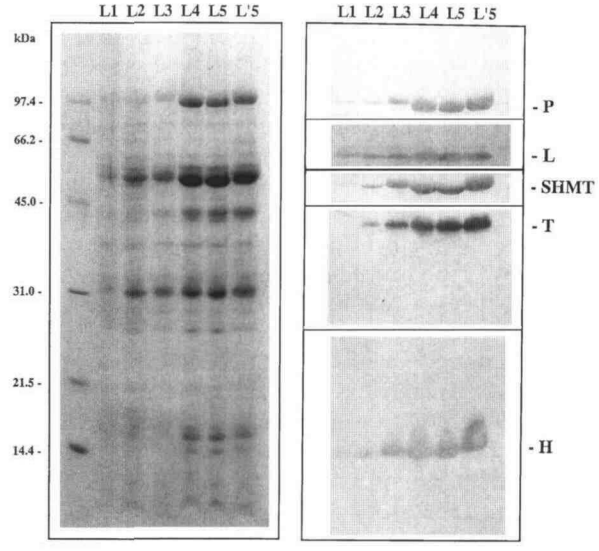

Figure 2. SDS-PAGE and immunoblot analysis of the proteins of GDC and SHMT during pea leaf development. L1 to L5 and L'5 refer to the developmental stages described in Figure 1. Mitochondrial proteins were resolved by SDS-PAGE (10–15% polyacrylamide) and stained with Coomassie brilliant blue R-250. Each lane contained 50 μg of mitochondrial proteins isolated from the leaves corresponding to the pea developmental stages (left). Western blot analysis of the same protein samples carried out with specific antibodies against P-, T-, H-, L-proteins of GDC and SHMT is shown on the right. Molecular mass values are indicated on the left.

Rubisco Expression during Pea Leaf Development

Since Rubisco and Gly decarboxylase are the key enzymes of the C_2 pathway of photorespiration, it was inter-

Table I. *Oxidation of Gly, NADH, pyruvate, and malate by mitochondria isolated from pea leaves at different developmental stages*

The different stages are defined in Figure 1. The values correspond to the average of substrate oxidation rates obtained from three to five different experiments and are expressed, with SE, in nmol O_2 consumed mg^{-1} mitochondrial protein min^{-1}, in the presence of ADP (0.1 mM).

Substrate	L1	L2	L3	L4	L5	L'5
Gly	3.2 ± 1.5	8.4 ± 2.9	9.5 ± 2.5	149 ± 11	201 ± 14	216 ± 16
NADH	245 ± 25	263 ± 21	244 ± 18	258 ± 28	363 ± 30	248 ± 27
Pyruvate	125 ± 11	113 ± 8	135 ± 15	112 ± 10	115 ± 15	75 ± 25
Malate	143 ± 18	186 ± 23	181 ± 20	134 ± 15	154 ± 18	108 ± 22

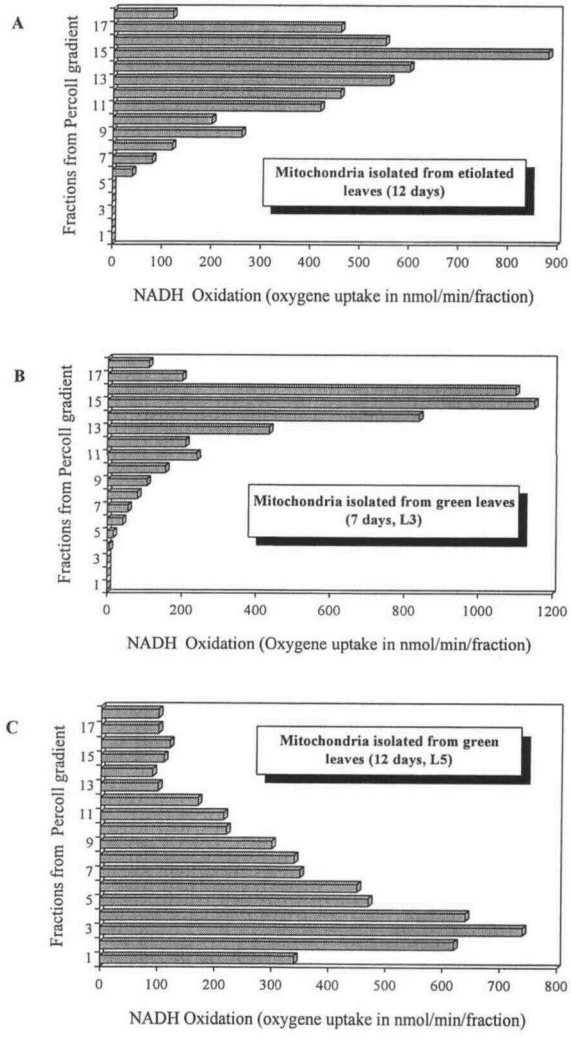

Figure 3. Variation in the density of mitochondria isolated from etiolated, young, and mature pea leaves. The three types of mitochondria were loaded separately on identical 28 to 42% Percoll gradients. After centrifugation the gradients were fractionated and mitochondria were detected in individual fractions by measuring NADH oxidation in the presence of ADP according to the method described in "Materials and Methods." The fractions are numbered on the left from the bottom of the tubes.

esting to follow the appearance of both complexes during the course of pea leaf development. Therefore, we isolated intact chloroplasts during pea leaf development at each stage defined in Figure 1 to monitor Rubisco expression during pea leaf development. It appears that chloroplasts isolated from the leaves of 4-d-old plants are able to incorporate CO_2 via the Rubisco at substantial rates (approximately 70 nmol CO_2 min^{-1} mg^{-1} chloroplastic protein). Furthermore, chloroplasts isolated from the leaves of 5-d-old plants exhibited CO_2 fixation rates almost identical to those measured with chloroplasts isolated from mature leaves (Fig. 4). The presence of Rubisco was confirmed by SDS-PAGE analysis. Indeed, Figure 4 shows that the small and large subunits of Rubisco are already present in the chloroplasts that were isolated from the leaves of 4-d-old plants. These results demonstrate that Rubisco appears well before GDC during the course of pea leaf development.

Expression of the Genes Encoding GDC and Rubisco during Pea Leaf Development

The steady-state levels of the mRNAs that encode the proteins of Gly decarboxylase were also analyzed during the development of the first leaf. Total RNA was isolated at each stage of development, and northern blot analysis was performed using the cDNAs encoding the H-, T-, P-, and L-proteins as probes. The gene patterns of expression that encode the specific proteins of GDC (H, T, and P) are similar (Fig. 5). The steady-state levels of the mRNA corresponding to these proteins increase progressively during the first days, with a peak of accumulation at stage L3, and decrease markedly after stage L4 (Fig. 5). The quantification of the signals reveals that at stage L1 the mRNA of the H-, T-, and P-proteins accounts for 25, 35, and 40%, respectively, of their maximum amount, which is measured at stage L3 for these three proteins (Fig. 5). Although there are some differences in these amounts, the overall induction of the expression appears to be coordinated for the H-, T-, and

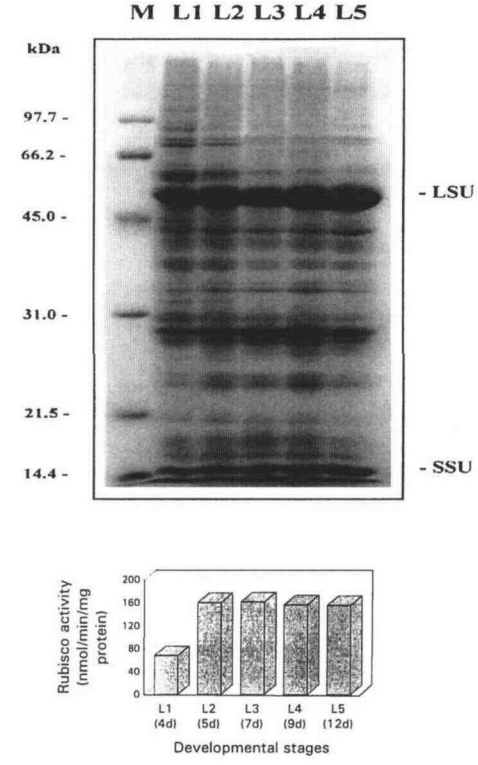

Figure 4. SDS-PAGE and Rubisco activity of chloroplasts isolated from pea primary leaves at different developmental stages. L1 to L5 refer to the developmental stages described in Figure 1. Chloroplastic proteins were resolved by SDS-PAGE (10–15% polyacrylamide) and stained with Coomassie brilliant blue R-250. Each lane contained 50 μg of proteins of intact chloroplasts. LSU and SSU indicate the positions of the large and the small subunits of Rubisco. M indicates the molecular mass standards. The lower part shows Rubisco activity in the same preparations of chloroplasts.

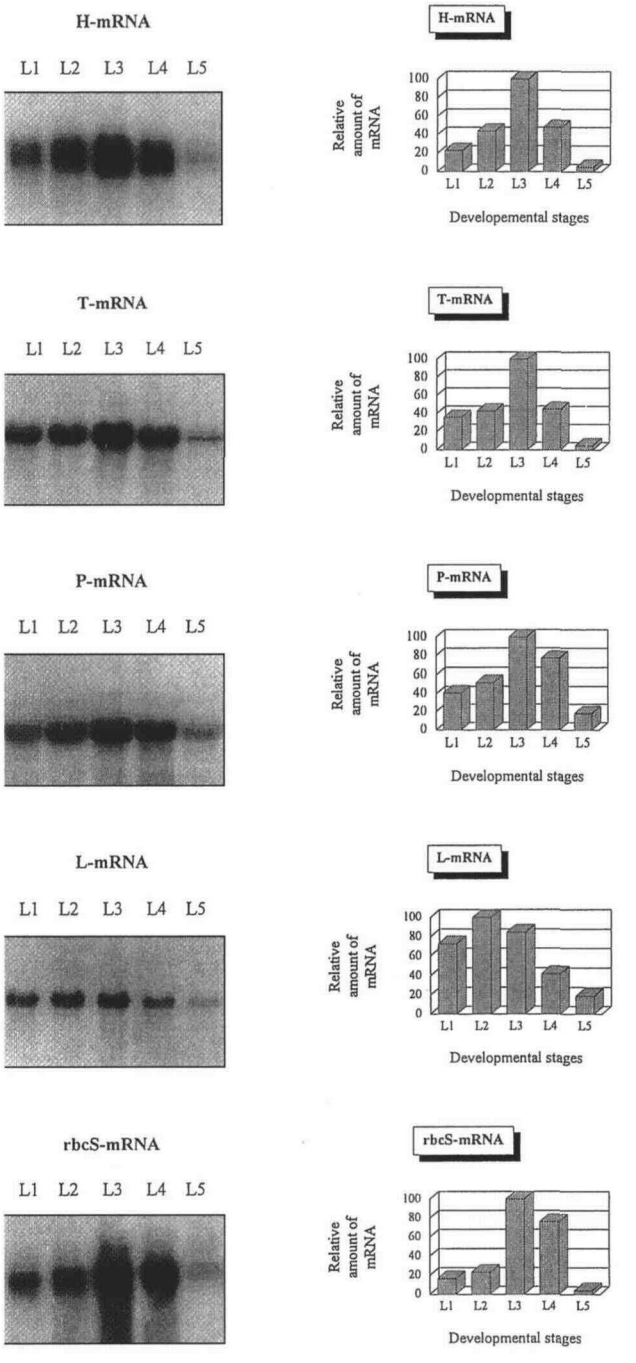

Figure 5. Northern blot analysis of mRNAs for GDC proteins and Rubisco small subunit protein during development of pea primary leaf. L1 to L5 refer to the developmental stages described in Figure 1. Equal amounts (20 μg) of total RNA were fractionated on a denaturing agarose gel, blotted onto a nylon membrane, and hybridized with specific labeled cDNA probes. Equal loadings of RNA were confirmed by subsequent methylene blue staining of the membrane. The right side of the figure shows the quantification of the signals performed with a phosphor imager apparatus (Molecular Dynamics). The amounts of mRNA are expressed relative to the maximum of radioactivity detected in the experiment.

P-proteins. In contrast, the mRNA of the L-protein during the first days, and in particular at d 4, is already abundant, since it represents 70% of the maximum intensity of the signal, which, in this case, is observed at stage L2 (Fig. 5). A careful comparison of Figures 2 and 5 indicates that the massive accumulation of Gly decarboxylase that was observed in the mitochondria at stage L4 does not coincide with the accumulation of corresponding mRNAs in the cytosolic compartment.

A similar northern blot analysis of pea leaf at the stages of development described in Figure 1 was performed using a cDNA probe that corresponded to the small subunit of Rubisco. For *rbcS* the pattern of expression is similar to what we observed for the mRNAs encoding the P-, T-, and H-proteins of GDC, with a peak of accumulation at stage L3 followed by a major decrease after stage L4 (Fig. 5). A comparison of Figures 4 and 5 indicates that, in contrast to the situation observed with GDC, there is no delay between the appearance of the mRNA for the small subunit of Rubisco and the synthesis of the protein.

DISCUSSION

GDC is present in large amounts in the leaf mitochondria of higher plants, which carry out C_3 photosynthesis. To obtain more information concerning the biogenesis of this complex and a better understanding of its regulation, we have examined the expression of the genes encoding the four proteins (P, T, H, and L) of GDC during the developmental cycle of the primary leaf of pea seedlings. We have shown that mitochondria isolated from the primary leaf start to oxidize Gly at substantial rates at stage L4 (9 d). In contrast, the oxidation rates for pyruvate or malate did not change significantly during the course of development. Our results show that Gly oxidation by mitochondria is correlated with the appearance of the proteins that are involved in the conversion of Gly into Ser, as judged by SDS-PAGE and western blot analyses. Considering the development of pea leaves, the appearance of Gly oxidation capacity seems to be closely related to the opening of the leaflets, which occurs after the 7-d stage, an event that allows the leaf to function as a morphologically efficient solar captor. We also observed that when mitochondria were isolated from the leaves of the second set (9-d-old plants), in which the leaflets were not opened, Gly oxidation was much lower than in expanded mature leaves (results not shown).

If we take into account the massive increase of mitochondrial protein due to the synthesis of GDC, we observe that exogenous NADH oxidation capacity is substantially higher at stages L4 and L5. There would seem to be a heterogeneous development of the mitochondria with respect to TCA cycle substrate oxidation and Gly and NADH oxidation. Lennon et al. (1995) have shown that Gly oxidation and alternative oxidase activities appeared simultaneously (around d 7) in pea primary leaf during development. However, the activity they report for the later stages is about five time less than the activity we observed in our experiments. This discrepancy in the Gly oxidation activities might be explained by the fact that the authors added

Cys to their grinding medium, which could inhibit GDC activity by reacting with pyridoxal phosphate of the P-protein and SHMT to yield a relatively stable compound containing a thiazolidine ring (Buell and Hansen, 1960; Douce et al., 1987).

We also noticed that the overall density of the mitochondria is strongly modified during pea leaf development: mitochondria that are isolated from pea leaves at the early stages of development, like those that are isolated from etiolated leaves, have a lower density than the mitochondria that are isolated from mature leaves. In fact, this is due to the accumulation of the GDC proteins and SHMT, which can reach 40% of mitochondria soluble proteins (Oliver et al., 1990), leading to an increase in the density because of the modification of the protein-lipid ratio. Consequently, it seems likely that the GDC proteins and SHMT fill preexisting mitochondria after the 7-d stage. Indeed, it was observed on the gradients that "light" mitochondria, which were found in the leaves of young plants, had nearly disappeared in mature leaves.

We analyzed the steady-state levels of the mRNAs corresponding to the proteins of GDC during pea leaf development and observed that the genes encoding the P-, H-, and T-proteins are expressed early (d 4) in a coordinated manner, with a peak of abundance at d 7. During this period there is a clear lack of correlation between the abundance of the mRNAs and the low representation of the peptides in the mitochondria. This situation strongly suggests that the expression of the genes that encode the specific proteins of GDC (P, T, and H) during plant development may be controlled posttranscriptionally. A translational control could occur either by modulating the rate of translational initiation, by sequestering mRNAs in translationally inaccessible messenger ribonucleoprotein particles, or by regulating the length of the poly(A) tail (for a review, see Curtis et al., 1995). Further experiments will be needed to confirm and elucidate the regulation mechanism. Furthermore, it is important, as has been shown by Turner et al. (1993), that once the GDC proteins are synthesized, their turnover must be low, since Gly oxidation activity remains high in the mature leaf, although the mRNAs become barely detectable.

The expression of the gene encoding the L-protein appeared to be regulated in a different manner than that of the three other proteins (P, T, and H) of the GDC, as has been observed previously in the case of wheat leaf development (Rogers et al., 1991). The difference between the expression pattern of the L-protein and that of the three other proteins of GDC is explained by the fact that a unique dihydrolipoamide dehydrogenase is involved in other mitochondrial multienzyme complexes, such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and branched-chain 2-oxoacid dehydrogenase (Turner et al., 1992; Bourguignon et al., 1992, 1996). In the early stages of development, the L-protein must be associated with other complexes. One of them is pyruvate dehydrogenase, as judged by the capacity of the mitochondria to oxidize pyruvate at this stage. After the 7-d stage, the amount of L-protein in the mitochondria increases and this is correlated with the appearance of the other proteins (P, T, or H) of GDC in the mitochondria.

The biogenesis of GDC during pea leaf development was compared with that of Rubisco. Chloroplasts were isolated from all development stages to monitor Rubisco activity. In contrast to GDC, which is not found in the early stages of development, Rubisco activity was high from the initial 4-d stage (42% of maximum activity). This was correlated with the number of Rubisco subunits detected by SDS-PAGE analysis. These results agree with the observation that initial transcription in pea plastids occurs 4 d after imbibition and reaches a maximum at 6 d (Dubell and Mullet, 1995). The analysis of steady-state levels of *rbcS* mRNA during pea leaf development revealed a pattern of expression similar to that of the P-, H-, and T-proteins of GDC. These results suggest that the genes encoding specific GDC proteins and photosynthetic genes such as *rbcS* follow a similar transcriptional regulation scheme, as proposed earlier by Kim and Oliver (1990). This is supported by the recent characterization of several light-responsive elements (GT boxes) in the promoter region of the *gdcH* gene in Arabidopsis (Srinivasan and Oliver, 1995). Thus, it appears that a major difference between the expression of GDC and Rubisco lies at the level of translation. Although the mRNAs for both enzymes follow similar patterns during the development of pea leaves, the biosynthesis of Rubisco starts several days before that of GDC. Therefore, it is likely that a posttranscriptional control must be involved in the expression of the genes encoding the P-, H-, and T-proteins.

Developmental regulation of photorespiration has been well documented using monocotyledonous leaves in which a gradient of development appears toward the tip of the leaf (Tobin et al., 1988, 1989; for a review, see Tobin and Rogers, 1992). It has been found that photosynthetic and photorespiratory enzymes are both located in the matured region of the leaf and follow the same gradient of differentiation and a similar spatial expression. However, in our study of the development regulation of GDC in pea primary leaf we found a lag time between the appearance of Rubisco and GDC that is possibly due to a translational control that is lifted when the leaflets open. The underlying physiological meaning is that during development, photorespiration appears only after photosynthesis has reached a level of activity that requires high amounts of photorespiratory enzymes to cope with the recycling of two carbon metabolites. We are currently working on the hypothesis that metabolites such as glycolate or Gly could be directly involved in the control of gene expression for photorespiratory enzymes. It is interesting that Sinclair et al. (1996) have recently shown that Gly induces the expression of the gene encoding the P-protein together with Gly decarboxylase activity in *Saccharomyces cerevisiae*.

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